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These results suggest that a specific subclass of excitatory amino acid receptor linked to PIns metabolism, or a phospholipase associated with the receptor, is transiently expressed in the neocortex during early postnatal development.

Postnatal Changes in Glutamate Stimulated Phosphoinositide Turnover in Rat Neocortical Synaptoneurosomes

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SUMMARY

Glutamate was found to stimulate the accumulation of ^{3}H - inositol phosphate in synaptoneurosomes prepared from rat neocortex during a narrowly defined period of postnatal development. No glutamate stimulation was observed on the day of birth, even though high levels of phosphoinositide (PIns) turnover were observed with the muscarinic agonist carbachol. Glutamate stimulated PIns turnover reached a maximum at one week of age, and decreased to adult levels by five weeks of age. Of the glutamate analogs tested, only ibotenate produced significant stimulation. N-Methyl D-Aspartate (NMDA) showed negligible stimulation and kainate showed only minor stimulation at the highest concentration tested (1 mM). Glutamate stimulation was not blocked by either the NMDA receptor antagonist 2-amino-5-phosphonovaleric acid (APV) or the broad spectrum glutamate antagonist kynurenic acid. These results suggest that a specific subclass of excitatory amino acid receptor linked to PIns metabolism, or a phospholipase associated with the receptor, is transiently expressed in the neocortex during early postnatal development.

KEY WORDS

Neocortex, Development, Plasticity, Excitatory Amino Acid, Phosphoinositide, Diacylglycerol, Protein Kinase C

INTRODUCTION

Recent evidence suggests that excitatory amino acid (EAA) receptors in the cerebral cortex not only mediate excitatory synaptic transmission, but also are involved centrally in the mechanisms of synaptic plasticity. The NMDA receptor, in particular, has received a great deal of attention for its apparent role in activity-dependent synapse modification at several locations, including the hippocampus [7,10] and the developing visual cortex[5,11]. Of special interest is the voltage-dependent calcium conductance mediated by this receptor[1,12,13]. A current working hypothesis is that calcium passing through the NMDA channel acts as an intracellular second messenger specifically to increase synaptic gain[4,5,21]. Little is known, however, about the involvement of other EAA receptors in synaptic plasticity. Recent work has shown that some EAA receptors are linked to the hydrolysis of phosphatidyl inositol-4,5-bisphosphate (PIP_2)[8,14,15,18]. Hydrolysis of PIP_2 by phospholipase C produces two second messengers[19]: inositol trisphosphate (IP_3) and diacyl glycerol (DG). IP_3 is believed to open channels within the endoplasmic reticulum, producing a rise in cytosolic calcium levels. DG is the

primary activator of calcium and phospholipid dependent protein kinase (PKC), whose activity has been associated with synaptic plasticity in the hippocampus[2].

The developing neocortex often has been used as a model for experience-dependent synaptic plasticity. We present here a possible biochemical correlate to this plasticity. Glutamate and one of its structural analogs, ibotenate, stimulate phosphoinositide (PIns) turnover in synaptoneurosome preparations from neocortex prepared from rats in a narrowly defined age range of 1 to 5 weeks postnatal. There is little stimulation in newborn or adult rats. This is in contrast to the stimulation of PIns turnover by the muscarinic acetylcholine receptor agonist carbachol which occurs throughout postnatal life.

MATERIALS AND METHODS

Synaptoneurosomes were prepared using the method of Gusovsky and Daly[9]. Briefly, male and female CD rats of various ages were killed, their brains removed, and the neocortex dissected free. The cortex was then weighed and homogenized in 10 volumes of oxygenated (95:5 O_2:CO_2) Krebs-Henseleit buffer, pH 7.4 (119 mM NaCl, 4.7 mM KCl, 1.18 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.18 mM KH_2PO_4,24.9 mM $NaHCO_3$, 10.0 mM glucose) in a glass-glass homogenizer with 4 strokes by hand. The homogenate was centrifuged at 1000 x g for 10 minutes and the supernatant discarded. The pellet was resuspended in 20 volumes Krebs-Henseleit buffer plus 50 mM HEPES (pH 7.4) and mixed with 1 μM ^{3}H-inositol (15 Ci/mmole). Aliquots of 320 μl were then incubated at 37° C for 60 minutes to label the inositol lipid pool. Twenty μl of 200 mM lithium chloride was added 10 minutes prior to the addition of agonists for a final incubation volume of 400 μl. The tubes were gassed with 95:5 O_2:CO_2 and capped before each incubation. After a 90 minute incubation, the suspension was centrifuged. The pellet was washed once with fresh buffer and resuspended in 6% trichloroacetic acid. After centrifugation, the

supernatant was collected and mixed with BioRad anion exchange resin AG 1-X8 (1 ml of a 50% slurry in water). The columns were washed 4 times with 1 ml water and 2 times with 1 ml 200 mM ammonium formate/100 mM formic acid to elute ^{3}H-inositol-1-phosphate (IP_1) into scintillation vials. All results consist of at least 3 experiments performed in duplicate and expressed as percent basal activity. The synaptoneurosomes used were unfiltered as previous reports have shown this step to make no difference in the results of this assay[9]. Also, since IP_3 is rapidly hydrolyzed to IP_1 in this system, accumulation of IP_1 is an accurate measure of overall phosphinositide turnover[9].

RESULTS

Addition of glutamate to synaptoneurosomes prepared from rats between 1 and 7 weeks of age resulted in a dose-dependent increase in the accumulation of ^{3}H- inositol-1-phosphate; maximal stimulation was usually obtained with 300 μM glutamate (Table I and Figure 1). The striking age-dependency of glutamate-stimulated PIns turnover is illustrated in figure 2. Stimulation (above basal) peaked at one week of age when it was ~6x the adult level. Glutamate-stimulated PIns turnover remained elevated substantially over the adult as late as 3 weeks postnatal; the adult value was not achieved until after 5 weeks of age (figure 2A). Unlike glutamate, 100 μM carbachol was effective in stimulating PIns turnover in newborn cortex (Figure 2B). And, although it also peaked at 1 week, carbachol stimulation decreased to the adult value by the third week postnatal.

Synaptoneurosomes from 3 week old rat cortex were prepared to identify the subtype of EAA receptor that is linked to PI metabolism. Our first approach was to determine whether stimulation of PIns turnover by 300 μM glutamate could be blocked by various EAA receptor

antagonists. Addition of the selective NMDA receptor antagonist 2-amino-5-phosphonovaleric acid (APV) at a concentration of 500 μM was ineffective in reducing glutamate stimulated PIns turnover (Table II). This suggests that the stimulation in neocortex is mediated by non-NMDA EAA receptors. Surprisingly, even the broad spectrum EAA receptor antagonist kynurenic acid (500 μM) was ineffective at blocking the stimulation by glutamate (Table II). These data indicate that glutamate stimulates PIns turnover at a site that is distinct from the EAA receptor types that have been distinguished by previously reported neurophysiological criteria.

We next attempted to address the question with glutamate receptor agonists. N-methyl-D-aspartate was used to stimulate "NMDA" receptors, kainic acid was used to stimulate "kainate" receptors, and both AMPA (α-amino-3-hydroxy-5-methylisoxazole-proprionic acid) and ibotenic acid were used to activate the "quisqualate" receptor subtype. The effects of these agonists in synaptoneurosomes from adult and 3 week old rats are shown in figure 3. In 3 week olds, agonists for the NMDA and kainate binding sites were generally ineffective in stimulating PIns turnover, although kainic acid showed

weak activity at 1 mM. Similarly, AMPA showed negligible ability to stimulate PIns turnover, even at very high concentrations. However, ibotenic acid proved to be a potent stimulant of PIns turnover in neocortical synaptoneurosomes from 3 week old rats. Significant stimulation of IP_1 acumulation occured with ibotenic acid concentrations as low as 10 μM. In adult rat synaptoneurosomes, agonists for NMDA and KA receptor types were also inactive. Ibotenic acid was able to stimulate PI turnover in synaptoneurosomes prepared from adult cortex. However, the maximal stimulation was approximately 1/3 that observed at 3 weeks (Figure 3A).

DISCUSSION

The results of these experiments indicate that the rat neocortex shows a robust ability to hydrolyze inositol lipids in response to glutamate during early postnatal development. These data also suggest that the stimulation by glutamate is associated with a unique glutamate receptor not described by many classification schemes. This receptor site appears to be sensitive to the "quisqualate" receptor agonist, ibotenate, but not to AMPA, a compound reported to be more selective for "quisqualate" receptors than quisqualate itself. Also, this receptor appears not to be blocked by kynurenic acid, a non-selective glutamate receptor antagonist. The receptor site in question may indeed be the "AP4" site previously described, as AP4 was found by Nicoletti *et al* [15] and Schoepp and Johnson[18] to block ibotenate stimulated PIns turnover in hippocampal slices.

Glutamate-stimulated PIns turnover in the neocortex appears to be limited to a period of postnatal development which, in the rat, extends from approximately 1 to 5 weeks of age. The decrease in glutamate stimulated PIns turnover in mature cortex is probably not the result of an increase in

the effectiveness of glutamate uptake systems because stimulation by ibotenate, which is not sequestered, also shows a marked age dependency (Figure 3). These data are consistent with the observations of Nicoletti *et al.*[14], who reported developmental changes in ibotenate stimulation of PI turnover in rat hippocampal slices.

The stimulation of PI turnover by glutamate has been observed in a number of different systems with somewhat varying results. Sugiyama *et al.*[20] showed that *Xenopus* oocytes injected with rat-brain mRNA could express a receptor which, when activated by glutamate or quisqualate, produced effects that resembled the physiological response to IP_3. These are likely to be the same "ibotenate" receptors responsible for glutamate stimulated PIns hydrolysis in neocortex and hippocampus. However, there appear to be additional types of EAA receptor that are linked to PIns turnover in certain brain regions. For example, Nicoletti *et al.*[16] have found that PIns hydrolysis is stimulated in primary cultures of cerebellar granule cells by NMDA and kainate as well as by quisqualate. Here, the stimulation by NMDA is antagonized by APV; stimulation by kainate is antagonized by γ-glutamylglycine. Our evidence suggests

that these receptor types are not linked to PI turnover in the rat neocortex.

Another receptor that is intimately associated with the hydrolysis of phosphoinositides is the muscarinic acetylcholine receptor. Because carbachol stimulated PIns turnover is substantial even at birth, we used it as a control condition to assess integrity of the assay system. As reported by Balduini *et al.*,[3] we found that carbachol also showed a large peak in its ability to stimulate the system at one week of age, but dropped to adult levels within three weeks (Figure 2). In marked contrast to this finding is the development of other markers of cholinergic activity[6]. Choline acetyl transferase activity, muscarinic receptors, choline uptake, and acetylcholine levels were all found to increase in neocortex from birth well into the fourth week of age. Balduini *et al.*[3] have suggested that the disparity between receptor number and agonist effectiveness in stimulating PIns turnover could be explained by developmental changes in the coupling of muscarinic receptors to phospholipase C.

Though glutamate stimulated PIns turnover has been shown to occur in astrocytes,[17] results from primary cultures of cerebellar granule cells show that the effect can

indeed occur in neurons[16]. If what we observe is mostly postsynaptic, then the data argue that during early postnatal development, EAA receptor activation stimulates two second messenger systems in parallel: calcium via NMDA receptors and DAG/IP_3 via the "ibotenate" receptor. Calcium has been proposed as a signal for increases in synaptic strength that accompany long-term potentiation in the hippocampus and neocortex. Products of phosphoinositide turnover could augment this effect via liberation of intracellular calcium and activation of PKC. Alternatively, this system could act in opposition to the NMDA system to weaken synaptic strength[5]. Indeed, the period between 1 and 5 weeks is when activity-dependent elimination of exuberant connections is a prominent feature of neocortical development.

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ABBREVIATIONS

AMPA	α-amino-3-hydroxy-5-methylisoxazole-proprionic acid
APV	2-amino-5-phosphonovaleric acid
AP4	2-amino-4-phosphonobutyric acid
EAA	Excitatory amino acid
IBO	Ibotenic acid
IP_1	Inositol-1-phosphate
IP_3	Inositol trisphosphate
KA	Kainate
Kyn A	Kynurenic acid
NMDA	N-Methyl-D-Aspartate
PIP_2	Phosphatidyl inositol-4,5-bisphosphate
PIns	Phosphoinositide
PKC	Calcium, phospholipid dependent kinase

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Stimulation of PIns turnover by various concentrations of glutamate at different ages.

Age (weeks)	Glutamate concentration (μM) 10	30	100	300	1000	3000
0	98±3	100±3	101±1	104±3	108±4	106±4
1	122±3	139±10	213±15	235±21	231±18	232±25
3	103±6	113±4	137±8	173±13	177±9	167±11
5	106±5	104±4	133±8	133±2	128±3	135±8
7	104±7	118±5	119±8	129±11	133±13	139±18
Adult	103±3	113±5	115±14	119±14	128±14	122±13

Table I. Accumulation of ^{3}H-inositol-1-phosphate in neocortical synaptoneurosomes prepared from rats of different ages. Results are means ± S.E.M. of at least 3 experiments, expressed as percent of basal phosphoinositide turnover.

Effects of glutamate receptor antagonists on glutamate stimulated PIns turnover in 3 week old rats.

Agent	% of stimulation by 300 μM Glutamate
+500 μM APV	99 ± 4 %
+500 μM Kyn A	100 ± 5 %

Table II. Levels of PIns turnover were determined for 300 μM glutamate ± 500 μM 2-amino-5-phosphonovaleric acid (APV) or kynurenic acid (Kyn A). Results are means ± S.E.M. and expressed as percent of stimulation by glutamate.

FIGURE LEGENDS

Figure 1. Glutamate stimulation of ^{3}H-inositol-1-phosphate accumulation in rat neocortical synaptoneurosomes prepared from 7 day old and adult rats. Values represent means ± S.E.M. of at least 3 experiments.

Figure 2. Agonist-stimulated phosphoinositide turnover as a function of age. A. 300 μM glutamate and B. 100 μM carbachol. Values are means ± S.E.M. of at least 3 experiments.

Figure 3. Effects of glutamate receptor agonists α-amino-3-hydroxy-5-methylisoxazole-proprionic acid (AMPA), N-methyl-D-aspartate (NMDA), kainic acid (KA), and ibotenic acid (IBO) on phosphoinositide turnover in synaptoneurosomes from: A. adult and B. 3 week old rats. Values are means ± S.E.M. of at least 3 experiments.

Figure 1

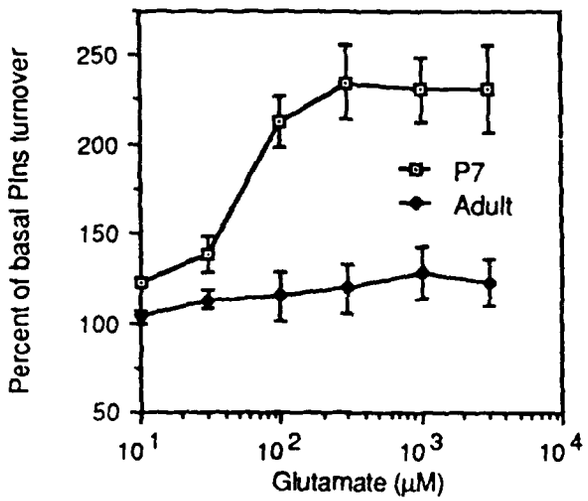

Percent of basal PIns turnover
250
200
150
100
50
10^1
10^2
10^3
10^4
Glutamate (μM)
P7
Adult

Figure 2

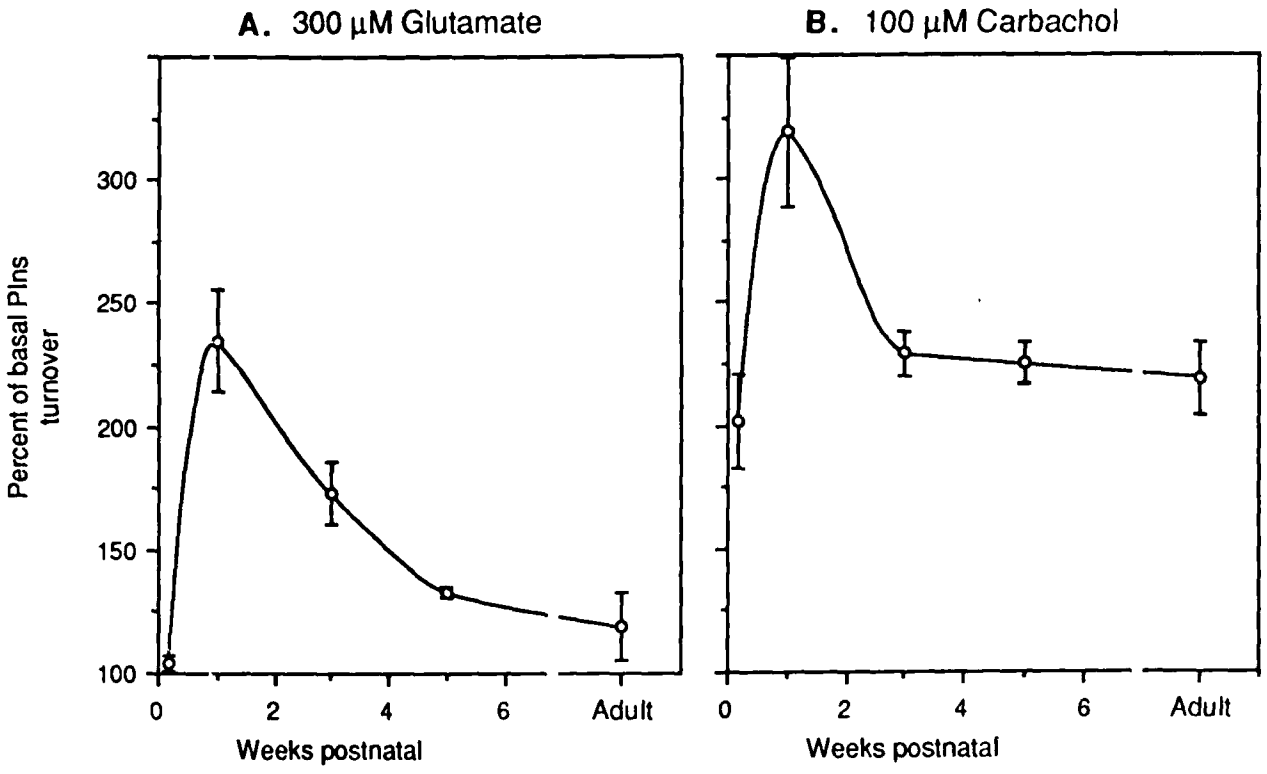
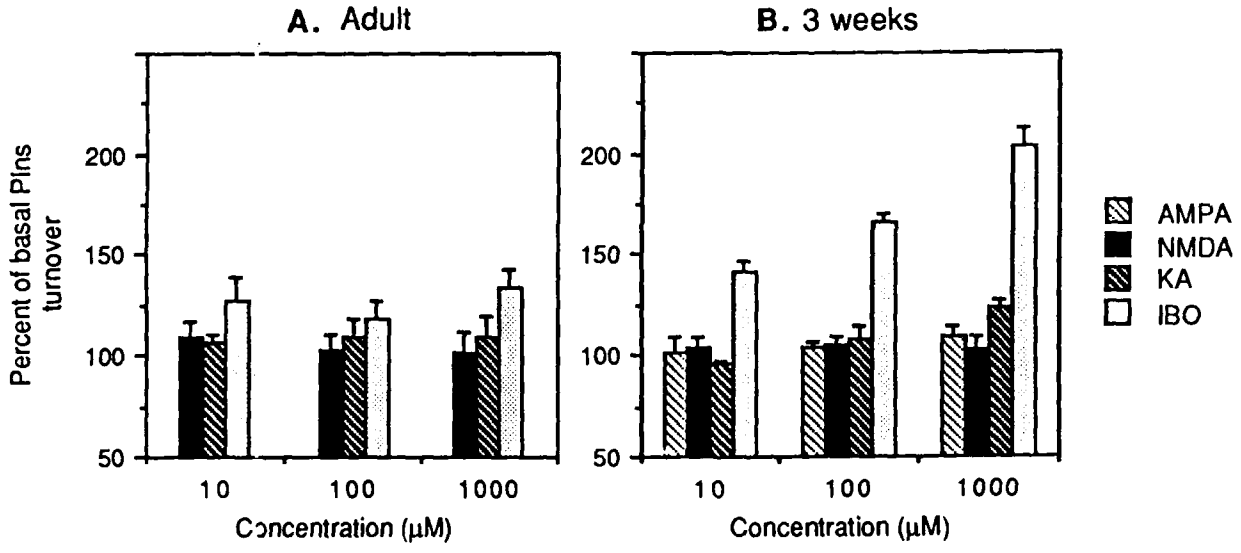

A. 300 µM Glutamate
B. 100 µM Carbachol
Percent of basal PIns turnover
300
250
200
150
100
0
2
4
6
Adult
Weeks postnatal
0
2
4
6
Adult
Weeks postnatal

Figure 3


A. Adult
B. 3 weeks
Percent of basal PIns turnover
200
150
100
50
10
100
1000
Concentration (μM)
AMPA
NMDA
KA
IBO